Withaferin A Targets Intermediate Filaments Glial Fibrillary Acidic Protein and Vimentin in a Model of Retinal Gliosis*§

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Gliosis is a biological process that occurs during injury repair in the central nervous system and is characterized by the overexpression of the intermediate filaments (IFs) glial fibrillary acidic protein (GFAP) and vimentin. A common thread in many retinal diseases is reactive Müller cell gliosis, an untreatable condition that leads to tissue scarring and even blindness. Here, we demonstrate that the vimentin-targeting small molecule withaferin A (WFA) is a novel chemical probe of GFAP. Using molecular modeling studies that build on the x-ray crystal structure of tetrameric vimentin rod 2B domain we reveal that the WFA binding site is conserved in the corresponding domain of tetrameric GFAP. Consequently, we demonstrate that WFA covalently binds soluble recombinant tetrameric human GFAP at cysteine 294. In cultured primary astrocytes, WFA binds to and down-regulates soluble vimentin and GFAP expression to cause cell cycle G0/G1 arrest. Exploiting a chemical injury model that overexpresses vimentin and GFAP in retinal Müller glia, we demonstrate that systemic delivery of WFA down-regulates soluble vimentin and GFAP expression in mouse retina. This pharmacological knockdown of soluble IFs results in the impairment of GFAP filament assembly and inhibition of cell proliferative response in Müller glia. We further show that a more severe GFAP filament assembly deficit manifests in vimentin-deficient mice, which is partly rescued by WFA. These findings illustrate WFA as a chemical probe of type III IFs and illuminate this class of withanolide as a potential treatment for diverse gliosis-dependent central nervous system traumatic injury conditions and diseases, and for orphan IF-dependent pathologies.

The overexpression of glial fibrillary acidic protein (GFAP) with vimentin is a hallmark of reactive gliosis in the central nervous system (CNS) (1, 2). These intermediate filaments (IFs) are expressed by reactive astrocytes and macro- and microglia during traumatic and inflammatory injury and in a range of CNS degenerative diseases (2). In fact, an enigma of major retinal diseases, including age-related macular degeneration, glaucoma, diabetic retinopathy, and retinopathy of prematurity, is retinal gliosis, for which there is no available clinical treatment (3–5).

Important fundamental insights on the structural and mechanical functions of IFs (6, 7) have now been validated in mouse lines deficient in type III IFs (2). These studies have illuminated that, whereas overexpression of vimentin and GFAP during CNS stress response and injury repair contributes to scar formation (8), their deficiency can be protective of tissue functions in certain contexts. For instance, pathogenic angiogenesis is impaired in vimentin-deficient (Vim KO) mice due to the decreased ability of newly formed blood vessels to cross the retinal inner limiting membrane in the model of hypoxia-induced retinal neovascularization (9). Interestingly, that study also identified in vimentin and GFAP double-deficient (Vim GFAP dKO) mice, and to a lesser extent in Vim KO mice, that the retinal ganglion layer is highly sensitive to mechanical stress, which was not observed in GFAP KO mice. Pathological neovascularization was also reduced in Vim KO mice in the corneal alkali injury model (10) and delayed vascularization in skin injury model (11), which is attributed to defective vascular endothelial cell integrity (12), because vimentin is the sole type III IF expressed in endothelial cells (13). On the other hand, Vim GFAP dKO mice subjected to spinal cord or brain injury recover favorably with improvement of glial scars (14). In fact, the complete absence of type III IFs in Vim GFAP dKO mice helps promote axonal regeneration and regain ambulatory function after spinal cord injury (15). These Vim GFAP dKO

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§§ The abbreviations used are: GFAP, glial fibrillary acidic protein; CNS, central nervous system; IF, intermediate filament; Vim KO, vimentin-deficient; Vim GFAP dKO, vimentin and GFAP double-deficient; GAPDH, glyceraldehyde phosphate dehydrogenase; PCNA, proliferative cell nuclear antigen; GS, glutamine synthetase; HSP, heat shock protein; INL, inner nuclear layer; TNF-α, tumor necrosis factor-α; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween 20; PBS, phosphate-buffered saline; LC-MS, liquid chromatography-mass spectrometry; MD, molecular dynamics; ULF, unit length filament; WFA, withaferin A; WT, wild type; HRP, horseradish peroxidase.
Witaferin A Targets GFAP

mice are also protected from retinal degeneration after experimental retinal detachment (16) and integration of transplanted cells and the extension of neurites in the host retinas of Vim GFAP dKO mice is favored compared with WT mice (17). Collectively, these reports provide substantive evidence that targeted down-regulation of the pathogenic overexpression of vimentin and GFAP in the CNS may be beneficial for injury healing in the CNS.

The natural product witaferin A (WFA) is a potent inhibitor of angiogenesis (18). WFA exerts it anti-angiogenic activity, in part, by directly targeting vimentin in vascular endothelial cells (10). This mechanism leads to vimentin fragmentation and down-regulation of its expression. Validating the importance of targeting this IF axis for anti-angiogenesis, we also demonstrated that the potent inhibition of corneal neovascularization by WFA in vivo is severely compromised in vimentin deficient (10). As our discovery identified that WFA binds tetrameric soluble vimentin by covalent modification of its unique cysteine residue (10), which is located in the conserved canonical rod 2B region of all type III IF proteins (19), we hypothesized that this residue (10), which is located in the conserved canonical rod 2B region of all type III IF proteins (19), we hypothesized that this critical WFA-druggable interaction site could also be exploited to perturb pathogenic overexpression of vimentin and GFAP in the CNS by developing a novel chemical probe of CNS trauma injury.

EXPERIMENTAL PROCEDURES

General Methods—WFA was purchased from a commercial vendor (Chromadex, Santa Ana, CA) and WFA-Bt synthesis has been previously described (10). All animal experiments were conducted in accordance with the Declaration of Helsinki, and procedures were approved by IACUC committee of the University of Kentucky.

Three-dimensional Model of the Human GFAP Tetramer Fragment—The homology modeling of the GFAP three-dimensional tetramer model and molecular docking for the WFA ligand binding were carried out as previously described (10). Also see supplemental data.

Astrocyte Primary Cultures Preparation and Treatments—Primary cultures of mouse astrocytes derived from WT and Vim KO mice were prepared from postnatal day 1 forebrain, as previously described (21). Also see supplemental data. For immunoblotting analysis, cells were plated at 70–80% confluency, previously described (21). Also see supplemental data. For immunoblotting analysis, cells were plated at 70–80% confluency, stimulated with 2 ng/ml of TNF-α for 18 h and treated with vehicle (Me3SO) or different concentrations of WFA for 2 h. Soluble cell fractions were extracted for Western blot analysis. For immunohistochemistry analysis, cells were cultured on poly-d-lysine 8-chamber slides and treated with vehicle or different concentrations of WFA for 18 h.

Cell Cycle and Apoptosis Analysis—Astrocytes were grown to confluence, growth arrested by serum starvation for 48 h, and treated with vehicle or WFA in the presence of serum for 21 h for apoptosis and cell cycle analysis as previously described (18).

In Vivo Corneal Injury Model—The alkali injury model of corneal neovascularization in Vim KO and WT mice in 129 Svev background has been described in detail previously (10). The simultaneous occurrence of retinal gliosis with corneal neovascularization was a discovery made during the course of this study.

Immunofluorescence—Using procedures previously described (10), astrocytes were fixed, permeabilized in 0.1 m phosphate-buffered saline (PBS) containing 0.2% Triton X-100 (PBS-T buffer), and blocked in 0.1 m PBS containing 5% goat serum, 1% bovine serum albumin, and 0.2% Triton X-100 (PBS-TS buffer) at 37 °C. Primary antibodies (see supplemental data Table S1) were diluted in antibody diluent solution (DAKO) and incubated with slides for 18 h at 4 °C. After washing, Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 594 goat anti-mouse were used as secondary antibodies. For immunostaining of Vim KO and WT mouse eye sections, 10-μm thin cryostat sections were collected, air-dried for 30 min, and fixed for 5 min. After washes in PBS, sections were permeabilized in PBS-T buffer for 1 h at 37 °C and then blocked for 1 h in PBS-TS buffer at 37 °C. After a brief wash, primary antibodies were diluted in DAKO solution and sections were incubated for 18 h at 4 °C and the secondary antibody was applied as described above. Digital images of immunostained sections were acquired on a Nikon TE2000 microscope at ×30 magnification.

Immunoblotting of Protein Extracts—Retinas were dissected on ice and homogenized in ice-cold lysis buffer containing Tris-buffered saline (TBS), 1% Nonidet P-40, 200 mM NaCl, and a protease inhibitor mixture. Samples were left on ice for 45 min and centrifuged at 14,000 × g for 5 min at 4 °C. The clear supernatants represented the soluble fraction, and the remaining insoluble fraction (pellet) was solubilized in Laemmli sample buffer. Protein extracts of the retina or cell cultures were subjected to Western blotting as previously described (10) using primary antibodies (see supplemental data Table S2). To reprobe blots, the membrane was incubated in aqueous-diluted (1:2 to 1:5) stripping buffer (250 mM Tris-Cl, 1.92 mM glycine, 1.0% SDS, pH 8.3) for 1 h, washed in TBS containing 0.2% Tween 20 (TBST), and re-blocked in TBST containing 5% non-fat dry milk before subsequent incubation with the next primary antibody. Blots were scanned and band intensities quantified using NIH ImageJ software and normalized to GAPDH or actin levels.

Isolation of WFA-Bt-binding Proteins by Affinity Chromatography—The WFA-Bt ligand binding studies in mouse astrocytes were performed as previously reported for endothelial cells (10). For retinal organ cultures, mouse eyes were freshly enucleated from Vim KO mice 7 days after corneal injury and cut sagitally into half. The posterior eye cup was placed in serum-free Dulbecco’s modified Eagle’s culture medium containing protease inhibitor mixture and excess WFA or vehicle for 1 h. WFA-Bt was subsequently added to the medium and incubated for 2 h. Retinal soluble tissue extracts were isolated as described above and biotinylated proteins affinity purified (10).

WFA-Bt Ligand Binding Studies with Recombinant Tetrameric GFAP—Purified soluble human recombinant GFAP (22) was incubated with vehicle or 5 μM WFA for 30 min at 37 °C. Subsequently, GFAP samples were incubated with 1 μM WFA-Bt for 1 h at 37 °C. Protein samples were boiled in Laemmli sample buffer containing 100 mM 2-mercaptoethanol
and subjected to SDS-PAGE and blotted. Blots were probed sequentially with anti-GFAP, stripped, and re-probed with streptavidin-HRP.

Liquid Chromatography-Mass Spectrometric (LC-MS) Analysis of GFAP—Purified recombinant human tetrameric GFAP was treated with 5 mM WFA or vehicle for 1 h and subjected to tryptic digestion. LC-MS analysis of tryptic peptides was acquired on a ThermoFinnigan LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Tandem mass spectra were acquired in a data-dependent manner. Five microscans were averaged to generate the data-dependent full-scan spectrum. The five most intense ions were subjected to tandem mass spectrometry, and five microscans were averaged to produce the MS/MS spectra. Masses subjected to the MS/MS scans were placed on an exclusion list for 30 s. The resulting MS/MS spectra were searched against human proteins in the Swiss-Prot data base with the XITandem search engine. The assignment of fragment ions in the MS/MS of the modified and unmodified peptides was obtained with the XITandem protein data base search engine allowing for a variable modification of +470 Da for the WFA adduct.

Image Analysis Software Development—The filament analysis software employed to measure GFAP polymer length is reported in the supplemental data.

Statistical Analysis to Filament Growth Curves—Comparisons between two polymer growth curves shown in Fig. 6, A–D, was made at representative percentiles (above the 50th percentile) using unpaired Student’s t test for statistical significance. A p value of less than 0.05 was considered significant. We also performed analysis of variance and non-parametric analysis of variance using the Wilcoxon procedure to compare WFA activity on the lengths of GFAP filaments in retinas of WT versus Vim KO-injured mice. We tested the hypothesis that the inhibitory activity of WFA in WT mice on GFAP polymer assembly cancels the stimulatory activity of the drug in Vim KO mice. The hypothesis could be stated as: (a–b) + (c–d) = 0, which is equivalent to (a+c) − (b+d) = 0 across the percentiles. The hypothesis is tested against the alternative that the effects do not cancel: a = WT mice vehicle-treated; b = WT mice WFA-treated; c = Vim KO mice vehicle-treated; d = Vim KO mice WFA-treated. The hypothesis is strongly supported as the p values are larger than 0.1.

RESULTS

The Binding Model of WFA with GFAP—Homology modeling of the GFAP tetramer structure was developed by using the previously constructed vimentin three-dimensional model as a template (10). Docking analysis of the molecular dynamics (MD)-simulated human GFAP-WFA complex revealed a stable binding mode for WFA in the binding pocket of tetrameric GFAP between the pair of head-to-tail α-helical dimers (Fig. 1B). As shown with the vimentin-WFA complex (10), the C3 and C6 carbons of WFA also lie in close proximity to the cysteine residue in the GFAP helix (Fig. 1C), permitting a nucleophilic attack by this thiol group on the electrophilic carbon centers of WFA (Fig. 1A). In addition, the carbonyl group of the steroidal moiety of WFA forms a hydrogen bond with the Gln297 side chain, although the hydrogen of the C4 α-hydroxyl group is stabilized by a salt bridge with Glu290 of the second monomer. Thus, superimposition of the binding site of GFAP-WFA on that of vimentin-WFA shows that the binding mode of the ligand is very similar in both tetramer fragments. This is evident in primary amino acid sequences that make up the WFA binding site having ~66% identity and 84% similarity between human GFAP and vimentin peptides (supplemental Fig. S1), and as well, these sequences are also evolutionarily conserved among vertebrate species (supplemental Fig. S2), which suggests strong functional relevance (23). Moreover, Asp331 and Val330 of vimentin are replaced by Glu297 and Leu296, respectively, in the GFAP fragment, giving rise to a small translation of the steroidal ring for WFA in GFAP due essentially to the higher steric interaction of the ligand and these GFAP residues (Fig. 1D). The MD trajectory revealed that position of the WFA compound from the initial structure was found to be stable after ~150 ps of the MD simulation (Fig. 1E).

Withaferin A Targets GFAP—We next tested the binding of WFA to vimentin and GFAP exploiting a cell permeable semi-synthetic biotinylated WFA analog (WFA-Bt) (10, 24) to isolate the WFA-binding targets from astrocytes. We show that WFA-Bt binds both vimentin and GFAP intracellularly in a WFA-competitive manner, and selectively isolates these targets from astrocytes by affinity chromatography (Fig. 2A). Because WFA-Bt binding to vimentin and GFAP was irreversible, being retained even under boiling conditions in 100 mM 2-mercaptoethanol as previously demonstrated for vimentin (10, 24), we postulated that WFA also binds GFAP covalently. Therefore, we investigated the biochemical interaction of WFA with soluble tetrameric GFAP using ligand-binding assays. We show that WFA-Bt binds to purified tetrameric human recombinant GFAP and this binding is prevented by free excess WFA. Importantly, the ligand-protein complex was retained under reducing conditions as demonstrated by probing the protein blots of this complex with streptavidin-HRP (Fig. 2B). Next, we queried whether the cysteine residue of GFAP was modified by WFA. WFA and vehicle-treated GFAP complexes were subjected to trypsin digestion and peptides were analyzed by liquid chromatography-mass spectrometry. We determined that WFA covalently modifies the 2B rod domain of GFAP at cysteine 294 (Fig. 2C), which corresponds to cysteine 328 of the homologous 2B rod domain of vimentin that is modified by WFA (10). These biochemical data confirm our molecular modeling results of the binding mode of WFA with tetrameric GFAP and extend WFA as a chemical probe of GFAP also.

WFA Targets Vimentin and GFAP Expression in Astrocytes—We employed an astrocyte primary cell culture model to inves-
tigate the effects of WFA in cells activated with TNF-α. WFA caused dose-dependent perinuclear condensation of vimentin and GFAP filaments in WT astrocytes at 0.5 μM and this effect was accentuated at 2 μM (Fig. 3A). However, with 4 μM WFA astrocytes also began to detach, an activity found to resemble the higher concentration effect of the 35-amino acid vimentin 1A peptide microinjected into fibroblasts (25). The remaining adherent WFA-treated astrocytes revealed large intense-staining vimentin aggregates that overlapped extensively with GFAP. In Western blot analysis both soluble vimentin and soluble GFAP expression were down-regulated considerably at 0.5 μM WFA, with the expression of vimentin being completely abrogated at 2 μM (Fig. 3B). Unlike the homogenous staining characteristics of WT astrocytes, Vim KO astrocytes revealed two phenotypes of GFAP staining cells. A majority (85–90%) of cells contained filamentous GFAP and the remaining cells expressed GFAP as short filament squiggles (26). The control cells showing both phenotypes is presented in Fig. 3C. WFA treatment at 0.5 μM affected primarily the GFAP squiggles, causing them to extend into an elaborate filamentous structure. At 2 μM GFAP staining was still filamentous and showed perinuclear condensation at 4 μM. GFAP staining started to become highly diffuse at 6 μM WFA, showing extensive cytoplasmic particulates (27) (Fig. 3C). Soluble GFAP expression assessed by Western blotting revealed very significant down-regulation of GFAP levels at WFA concentrations between 0.5 and 4 μM, and interestingly, at 6 μM the reappearance of soluble GFAP in detergent extracts was apparent (Fig. 3D). Thus, the effect of WFA on GFAP particulate/aggregate formation in WT and Vim KO astrocytes differed mainly in the dose of WFA required to elicit this response and that the particulate size appeared to be larger in WT astrocytes.

WFA Induces Vimentin-mediated Cell Cycle Arrest in Astrocytes—We next investigated the activity of WFA on the regulation of astrocyte cell cycle. Astrocytes were growth arrested and induced to proliferate with serum in the presence...
and absence of WFA. WFA potently blocked the proliferation of WT astrocytes in G0/G1 in a dose-dependent manner (Table 1). On the other hand, serum-starved Vim KO astrocytes remained restricted in G0/G1 (90.5%) unresponsive to serum stimulation (see also supplemental Fig. S3) and thus did not display any significant response to drug treatment. The lack of growth response was not due to reduced cell viability, because WFA-induced apoptosis was evident in Vim KO astrocytes only at the highest concentration of 2.4 μM WFA (supplemental Fig. S4). Taken together, these results identify WFA as a potent growth inhibitor causing cell cycle arrest in WT astrocytes that is mediated primarily by down-regulation of soluble vimentin. Down-regulation of GFAP by WFA in Vim KO astrocytes instead initiates apoptosis when drug concentrations exceed the cytostatic dose (less than 2 μM). This differential cell cycle inhibitory mechanism of WFA in WT astrocytes prompted us to investigate the targeting of vimentin and GFAP WFA in vivo in the pathophysiological environment of retinal gliosis.

**WFA Down-regulates Soluble Vimentin and GFAP Expression in Reactive Müller Glia**—We employed a corneal alkali injury model that was originally developed to induce corneal neovascularization (20). We adopted this model to investigate retinal gliosis because overexpression of GFAP and vimentin in reactive Müller glia captures hallmark features of gliosis (8). Vimentin expression in uninjured control mice is found in ganglion astrocytes and Müller glia (16) (Fig. 4A), paralleling the staining pattern of desmin (supplemental Fig. S5) that marks the radial processes of Müller cells in normal retina (28). GFAP staining in uninjured mice localizes only to ganglion astrocytes (Fig. 4D). Upon injury, GFAP expression was strongly up-regulated in retinal ganglion astrocytes and Müller glia with extensive overlap of staining with vimentin-con-

![Figure 2](http://www.jbc.org/)

**FIGURE 2.** WFA binds vimentin and GFAP in astrocytes and modifies cysteine 294 of recombinant human GFAP. A, astrocytes were incubated with 20 μM WFA or vehicle for 30 min and subsequently with 5 μM WFA-B for 2 h. Soluble cell lysates were affinity purified over Neutravidin-agarose columns (10) and subjected to SDS-PAGE and blotted. Protein blots were probed sequentially with anti-vimentin (V9) and anti-GFAP antibodies and last with streptavidin-HRP (Strep-HRP). The endogenous 70-kDa biotinylated protein served for equal protein loading. The asterisk marks the 56-kDa vimentin and the arrowhead the position of GFAP. B, recombinant human tetrameric GFAP was preincubated with vehicle or 5 μM WFA and subsequently with 1 μM WFA-B. Protein samples were boiled and subjected to SDS-PAGE and blotted. The blot was probed sequentially with anti-GFAP antibody and then with Strep-HRP. C, recombinant human tetrameric GFAP was incubated with vehicle (Veh) or 5 μM WFA and tryptic digests were subjected to analysis by liquid chromatography-mass spectrometry. The MS-MS scans of the peptide chromatograms for the 13-amino acid tryptic peptide, QLQS-LTCDELSSLR, shows an increase by the mass of WFA (+470.27 daltons) at the b7- and y7- fragment ions, which is the cysteine residue.
Withaferin A Targets GFAP

VOLUME 285 • NUMBER 10 • MARCH 5, 2010

mice revealed similarity to GFAP staining of vehicle-treated
ments (Fig. 4K). In close similarity, the retinas of injured
iform layer (Fig. 4F). Interestingly, the presence of ~2-μm long GFAP
stained as dots was observed throughout the retinas of uninjured Vim KO mice (Fig. 4L).

The differential solubility of tetrameric IFs from the insoluble polymeric forms was exploited to biochemically character-
ize vimentin and GFAP expression. We found in retinas of WT-
jured mice that soluble vimentin is substantially overexpressed as a major 56-kDa species, and WFA treatment down-regulated its expression (Fig. 5A). In the insoluble frac-
tion, although injury caused increased expression of the single major 56-kDa species, WFA treatment did not affect its expres-
sion (Fig. 5B), most likely due to substantial contribution from pre-existing retinal and choroidal vasculature. GFAP expres-
sion was more complex; three major soluble GFAP species (38–50 kDa) were overexpressed in response to injury in WT mice compared with control uninjured tissue that expressed low levels of two species (Fig. 5A). However, in retinas of injured Vim KO mice, multiple GFAP species within this size range were overexpressed as soluble forms (Fig. 5A). This corre-
sponds to certain intermediate sized insoluble GFAP species in Vim KO retinas being substantially down-regulated (Fig. 5B).

These findings are in agreement with the key role of vimentin as a facilitator of heteropolymeric filament assembly, which is compromised in vimentin deficiency (29) and corroborates the accumulation of multiple soluble GFAP species (Fig. 5A). Treatment of WT mice with WFA caused a reduction in levels of all soluble GFAP species, whereas, in Vim KO mice both drug-sensitive and -resistant soluble GFAP species were observed (indicated by arrows in Fig. 5A). Interestingly, in Vim KO retinas the activity of WFA also resulted in detection of novel lower molecular mass (~35–37 kDa) insoluble GFAP species (Fig. 5B, arrows) that were not observed in drug-treated WT retinas.

We next assessed the activity of WFA on other retinal pro-
teins; the expression of the WFA alternate target annexin II (30) did not change in response to retinal gliosis or with WFA treatment (Fig. 5A), and so also, the expression of glutamine synthetase (1, 4) and cellular retinaldehyde-binding protein (31) was not affected by injury or WFA treatment (Fig. 5A). However, cellular retinaldehyde-binding protein distribution within Müller cells mimicked localization of GFAP expression (32) in both genetic deficiency of vimentin and WFA-induced target knockdown (supplemental Fig. S7). Finally, expression of heat shock protein (HSP)-70 was also unaltered among the different treatment groups (Fig. 5A), which suggests an absence of protein toxicity in this injury model. This finding contrasts that found in transgenic mice overexpressing mutated vimentin where misfolded vimentin is found to trigger the up-regulation of HSP-70 due to protein aggregation toxicity (33).

WFA Binds GFAP in Vivo and Stimulates Polymer Assembly in Vimentin Deficiency—We next assessed Vim KO mice to confirm the direct binding of WFA to GFAP in vivo during gliosis. Vim KO mice were subjected to the corneal alkali injury and 7 day post-injured eyes were isolated, cut in half, and the posterior section eye cup was placed in organ culture to isolate WFA-binding proteins using Neutravidin affinity purification (10). We show that incubation of retinas with WFA-Bt isolates several GFAP species that bind WFA in a competitive manner.

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<td><strong>Cell cycle analysis of WT and Vim KO astrocytes treated with WFA</strong></td>
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FIGURE 3. **WFA down-regulates vimentin and GFAP expression in astrocytes.** A, WT mouse astrocytes were treated with WFA or vehicle for 2 h and stained for GFAP and vimentin expression with counterstaining with 4',6-
diamidino-2-phenylindole (merge). The arrowheads show the extensive aggregation of vimentin and GFAP IFs at high WFA concentration. The bar in all panels represents 25 μm. B, in other experiments, WT astrocytes were activated with 2 ng/ml of TNF-α for 18 h and subsequently treated with WFA or vehicle for 2 h. Soluble lysates were subjected to SDS-PAGE, protein blotted, and probed sequentially with antibodies to vimentin, GFAP, and β-actin. C, Vim KO astrocytes were subjected to similar experiments as in A and stained with antibody against GFAP. The bars in the square panels are 25 μm, whereas those of the magnified images in circular panels are 5 μm. D, Vim KO astrocytes treated with WFA as in B were subjected to Western blotting and probed with antibody against GFAP.
geted by WFA in Vim KO retinas (Fig. 5B, arrows) compared with WT retinas. Second, the expression of ganglion-associated 50-kDa insoluble GFAP species (Fig. 5B, arrowhead), present in uninjured control retinas (Fig. 4D), was largely unaltered by injury or WFA treatment. Using the data set comprising ~17,000 individual GFAP filaments, we derived the percentile distribution for filament size for each treatment group. These data were employed to develop GFAP polymer growth curves (Fig. 7, A–D). Interrogation of the growth curves (Fig. 7A) showed that GFAP filaments in WT retinas are the longest, whereas those in Vim KO retinas are the shortest; differences become recognized above the 50th percentile (Fig. 7E). Second, WFA treatment caused inhibition of GFAP polymer assembly in retinas of injured WT mice (Fig. 7B), which also became significant at the 75th percentile (Fig. 7E). However, it came as a surprise to find that WFA treatment induced GFAP polymer assembly in injured Vim KO retinas (Fig. 7C), which was significant even at the 50th percentile (Fig. 7E). Furthermore, we discovered that the GFAP polymer assembly profiles of WT mice treated with WFA overlapped considerably with WFA-treated Vim KO mice over the entire filament formation process (Fig. 7D). This observation was tested as a hypothesis (see “Experimental Procedures”), which allowed us to conclude that these curves indeed overlap (analysis of variance p = 0.3158; Wilcoxon test p = 0.4458).

Collectively, these data suggest that WFA-induced down-regulation of vimentin expression leads to deficits in GFAP polymer assembly, and this functional outcome is counterbalanced by the induction of the WFA GFAP polymer assembly in the genetic deficiency of the major target of WFA, vimentin.

**Withaferin A Targets Cyclin D3 and p27**

*Withaferin A Targets GFAP*

We investigated the expression of cyclin D3 and cyclin-dependent kinase inhibitor p27\(^{kip1}\), which are two critical cell cycle regulators of Müller glia (32). In control WT mice, expression of cyclin D3 was found in the cell bodies of the inner nuclear layer (INL) that contain Müller nuclei and in ganglion astrocytes (32). Upon

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**FIGURE 4.** **WFA interferes with GFAP filament formation during retinal gliosis.** WT and Vim KO mice were subjected to the corneal injury model and treated with 2 mg/kg/day of WFA or with vehicle (Veh) by intraperitoneal injection for 7 days. Tissue sections of eyes from uninjured (Cont) and injured mice (n = 4/group) were stained with antibodies to vimentin (red), GFAP (green), and 4',6-diamidino-2-phenylindole (blue) and assessed by epifluorescence microscopy. The fluorescence overlaps are shown (G, H, I, M, N, and O). The bar in representative retinal images (A–O) is 20 μm. The squiggles identified as tiny dots are marked by asterisks (L). GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Data are representative of two independent experiments (n = 36 mice). Intra-retinal blood vessels staining for vimentin (C) was corroborated by staining for endothelial cell marker CD31 (supplemental Fig. S6).

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Fig. 6). Interestingly, the higher molecular mass ~50 kDa abundant soluble GFAP species showed the greatest level of sensitivity to WFA competition (Fig. 6B), which was similar to the pharmacological activity of WFA in Vim KO mice (Fig. 5A). To probe for subtle filament characteristics among the different groups we developed a computer software program to analyze GFAP-stained IFs in tissue sections (supplemental Fig. S8). We confined the filament analysis to radial Müller glia, first, because injury-induced expression of GFAP localizes to Müller cells (4, 8) (Fig. 4E), and several newly detected insoluble GFAP species induced during gliosis were differentially tar-
chemical injury, cyclin D3 expression increased most prominently in the INL, an expression pattern that was potently down-regulated by WFA treatment (Fig. 8A). In control Vim KO mice, cyclin D3 expression was also found in Müller glia, and with injury, cyclin D3 expression was overexpressed in the INL and ganglion astrocytes with WFA treatment. On the other hand, injured WT mice down-regulated p27Kip1 in Müller glia and ganglion cell layer compared with control retinas, and this pattern of expression was reversed by WFA treatment (Fig. 8B). In uninjured Vim KO retinas p27Kip1 expression was also detected in the Müller glia and ganglion cell layer, however, p27Kip1 expression was potently down-regulated with injury. WFA treatment up-regulated p27Kip1 expression in Müller glia.

To relate the effects of WFA on the regulation of these cell cycle mediators on cell proliferation we also analyzed the expression of proliferating cell nuclear antigen (PCNA) by Western blot analysis (Fig. 8C). PCNA expression was induced with injury in both WT and Vim KO retinas, however, WFA treatment down-regulated PCNA expression in WT mice, whereas the injury-induced expression level of PCNA was sustained in Vim KO retinas. Collectively, these findings demonstrate that WFA differentially affects the expression of critical cell cycle growth regulators in the retinas of injured WT and Vim KO mice.

DISCUSSION

The type III IFs have until now remained elusive to small molecule perturbation in vivo due, in part, to their dynamic expression, elaboration as multiple species/variants, and bearing no definable druggable site in their soluble tetrameric or higher order polymeric structures (6, 19). Using chemical genetic approaches coupled with affinity purification strategies, we overcame this challenge with the discovery that WFA binds at a novel protein-protein interface of tetrameric vimentin (10). In this article, we now demonstrate that WFA independently also binds in a similar fashion to soluble tetrameric GFAP to
Withaferin A Targets GFAP

FIGURE 7. WFA modulates GFAP polymer assembly in vivo. WT and Vim KO mice were subjected to the alkali injury model (n = 16) and treated with 2 mg/kg/day of WFA or vehicle for 7 days. Tissue sections were stained for GFAP and digital fluorescent images from each group (n = 16 to 25) were obtained using a ×30 objective on a Nikon TE2000 microscope. The images were analyzed in a double-blinded manner to measure filament length. The percentile distribution of GFAP filaments in each group was plotted against their filament length and polymer growth curve comparisons shown (A–D). E, data at selected percentiles showing GFAP filament length for each group and Student’s t test applied to selected percentile comparisons.

TABLE 1

<table>
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<tr>
<th>Percentile</th>
<th>WT/Veh (μm)</th>
<th>WT/WFA (μm)</th>
<th>KO/Veh (μm)</th>
<th>KO/WFA (μm)</th>
<th>A* P value</th>
<th>B* P value</th>
<th>C* P value</th>
<th>D* P value</th>
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<td>7.5</td>
<td>9.5</td>
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<td>&lt;0.0001</td>
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<tr>
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<td>3.5</td>
<td>3.5</td>
<td>4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.9788</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

A* = WT/Veh vs KO/Veh; B* = WT/Veh vs WT/WFA; C* = KO/Veh vs KO/WFA; D* = WT/WFA vs KO/WFA

Most cells retain relatively small amounts of soluble tetrameric oligomers of vimentin and GFAP compared with their polymerized filamentous forms. Under normal conditions, Müller glial cells also maintain high levels of filamentous vimentin, but that of GFAP is not detectable, however, being abundantly expressed in the retinal ganglion layer astrocytes (40). This IF distribution is found clearly altered in Vim KO mice, revealing high levels of soluble GFAP in the retina, an expression pattern previously reported also in brain tissues of Vim KO mice (29). Moreover, GFAP expression in the Vim KO mouse retina is further elaborated as a complex profile of soluble isoforms. Thus, despite previous investigations of Vim KO retinas (9), our finding that GFAP squiggles are expressed in uninjured Vim KO retinas starting below the ganglion cell layer and extending into the outer nuclear layer is novel, and this is consistent with squiggles being produced in vimentin-deficient cells (27, 34). This retinal staining pattern has now been validated in several progeny from over 5 breeding colonies of Vim KO mice. Hence, our conclusion is that even under normal situations GFAP tetramer-polymer homeostasis is markedly altered in Müller glial cells of adult mice deficient in vimentin, but no defining physiological defect is apparent due to this alteration. However, these filament precursors in response to injury assemble into only short GFAP filaments that reach lengths less than half the size of those found in injured WT mice. One might anticipate that such phenotypes result from impediments in the IF polymerization process, which is believed to be a highly regulated three-step process (41, 26). Importantly, our findings also underscore a critical role for vimentin as a direct or indirect mediator of GFAP assembly. The filament assembly first step entails the lateral annealing of tetramers into unit length filaments (ULFs) with longitudinal annealing of two ULFs, followed by a second step involving the longitudinal annealing of an ULF to the end of a filament and the final step of end-to-end annealing of two filaments. The detection of ULFs as dots and their fusion products that give rise to squiggles has been now confirmed also in vivo (35). On the one hand, our findings showing a dispersion of 2–2 μm long GFAP particle-like structures in control Vim KO retina, which are considerably larger than 60 nm ULFs (41), would argue that GFAP precursors have undergone the initial step of filament assembly to form squiggles. However, these squiggles cannot elongate past the 2-μm restriction size due to a possible impediment in the longitudinal annealing of an ULF to a filament. On the other hand, injured Vim KO retina that can produce short filaments of 23–25 μm would implicate a defect also in the target its down-regulation, which affords this natural product as a novel chemical probe of reactive gliosis in the CNS.

The selective targeting of soluble vimentin and GFAP WFA manifests in a dose-dependent perturbation of their polymeric structures in astrocytes. We previously reported that low concentrations of WFA were well tolerated in vimentin polymerization assays, whereas high concentrations of WFA caused filaments to aggregate (10). The biological relevance of this mechanism in WT astrocytes is reflected in the structural perturbation of vimentin and GFAP filaments leading to their perinuclear condensation at WFA concentrations between 1 and 2 μM, whereas filament aggregation becomes the major phenotype at concentrations exceeding 4 μM. These findings are similar to our observations of the effects of WFA in endothelial cells (10), except that these latter cells exhibit a higher level of sensitivity to WFA. Thus, it was unanticipated that in Vim KO astrocytes the principal effect of low WFA concentrations is to facilitate the extension of short GFAP squiggles into their long filamentous forms. These GFAP squiggles found in Vim KO astrocytes have been previously reported (34) and resemble the IF precursors described in spreading cells (35). Moreover, this phenomenon reflects a functional deficiency of vimentin (36), because transfection of GFAP into vimentin-deficient SW13 cells or into Vim KO astrocytes, but not WT astrocytes, was also reported to produce the short GFAP squiggles (27). To our knowledge, ours is the first report of a small molecule that binds GFAP and promotes its self-assembly at low concentrations in vimentin-deficient astrocytes and Müller glia in vivo. However, the dose-related perinuclear condensation and aggregation phenotypes produced at higher WFA concentrations in WT astrocytes mimic similar affects produced in cells when injected with the filament assembly perturbing vimentin 1A peptide (25) or a synthetic peptide from the coil 2b region or also with an anti-2b antibody (37). These findings would suggest that IF perturbation can produce different biological effects in cells, which depend on several factors including dose of drug (18), the cellular or pathological context of cells that determines their post-translational status (38), and conformational geometry of the cysteine residue that is preferentially targeted by cysteine-reactive small molecules when vimentin is in its soluble tetrameric form compared with the polymeric form (39).
end-to-end annealing step between two GFAP filaments. Thus, these data implicate vimentin as a regulator of three key aspects of GFAP expression homeostasis and filament equilibrium in Müller glial cells. The first is to restrict GFAP expression at a transcriptional or post-transcriptional level. The second is to prevent the promiscuous self-assembly of soluble GFAP into polymers. The third is to facilitate the polymerization of GFAP by promoting filament end-to-end annealing in response to stressors during retinal gliosis. In its first role as a regulator of soluble GFAP expression, it was previously debated that vimentin may control GFAP expression at a transcriptional step (40, 42). However, the ~2-fold increase in soluble GFAP in brain tissue of Vim KO mice was not found to be related to increased GFAP mRNA levels (34), instead, these authors rationalize that soluble GFAP exists as a steady-state precursor pool of cytoskeletal GFAP (43), which may also be the case in Müller glia. In its purported second role, vimentin could become a co-partner with soluble GFAP subunits for heteropolymerization, and hence, a deficiency of vimentin would manifest in an impediment in GFAP polymerization. This inference is speculated for Müller glial cells also based on findings which show that vimentin and GFAP can partner as heteropolymers in astrocytes (29), an argument rationalized by us here and others who have found impaired GFAP assembly in vimentin-deficient astrocytes and mice (34). Alternatively, our observations made in Vim KO retinas may also be explained by additionally invoking the role of a vimentin-binding co-factor such as plectin (44). For instance, soluble vimentin rapidly forms into long filamentous polymers in plectin-deficient fibroblasts without displaying the ~2-μm long squiggle precursors found in WT cells (44). These authors have rationalized that plectin binds to the ends of such vimentin squiggles and modulates vimen-
tin polymer assembly (44). Moreover, because plectin can regulate the spreading of GFAP filaments by shifting soluble GFAP to insoluble polymeric forms (36), one may derive a rationale that Vim KO mice could have down-regulated expression levels of plectin. However, this has not been reported and remains to be determined whether plectin expression is altered in Vim KO mice.

It is widely known that mitotic cell cycle kinases phosphorylate GFAP and vimentin at specific amino acid residues (38), which leads to their disassembly and subsequent conversion into soluble tetrameric oligomers (40). Thus, WFA incorporation into vimentin polymers could sensitize WFA-bearing filaments to one or more IF-kinases that can cause their phosphorylation-induced disassembly. This WFA-induced vimentin knock-out in alkali-injured WT mice would emulate the genetic deficiency of vimentin producing short GFAP filaments. However, this inhibitory activity is partially compensated by the direct binding of WFA to GFAP that promotes IF assembly. Hence, GFAP filaments in WFA-treated WT mice are longer than those of vehicle-treated Vim KO mice, but reach equivalent lengths to those in WFA-treated Vim KO mice because of the absence of the vimentin-mediated inhibitory activity. However, the vimentin- and GFAP-directed opposing activities produced by WFA are not of equivalent potency because GFAP filament lengths in WT mice treated with WFA are shorter than those compared with vehicle-treated mice. The preferential targeting of higher molecular weight GFAP species by WFA in Vim KO retinas may reflect their differential post-translational status, such as phosphorylation as gleaned from the multiple GFAP isoforms expressed in Vim KO retinas. Alternatively, these WFA-sensitive GFAP isoforms may be alternative spliced GFAP variants that are generated in response to perturbation of GFAP homeostasis in vimentin deficiency. The speculated presence of these alternate spliced GFAP variants, which are inhibitory to polymerization (45) could limit self-assembly of GFAP at the squiggle stage in uninjured retinas and the end-to-end annealing step in stressed retinas when their stoichiometric levels exceed a certain threshold compared with GFAP-α levels. Consequently, the abrogation of these inhibitory GFAP isoforms of WFA would help promote longer filaments. Importantly, these major biochemical perturbations of GFAP equilibrium observed in vimentin deficiency and harmonized by WFA treatment are quite selective, because expression levels of other retinal proteins, including glutamine synthase, cellular retinaldehyde-binding protein, and HSP-70, and the alternate GFAP-binding protein annexin II (30), remain unchanged with drug treatment.

The down-regulation of soluble vimentin and GFAP of WFA in reactive Müller glia of injured WT mice parallels the potent targeting of these soluble IFs in WT astrocytes by nanomolar concentrations of WFA that results in $G_{0}/G_{1}$ cell cycle arrest. Consequently, IF targeting of WFA to cause inhibition of expression of cyclin D3 in Müller glial cells is corroborated by down-regulation of injury-induced PCNA expression in WT mice. Indeed, cyclin D3 is a key $G_{1}$-cell cycle protein known to be expressed in proliferating Müller glia cells (46), and whose down-regulation is believed to ultimately contribute to keeping Müller glial cell proliferation in check (32). The cell cycle inhibitory mode of action of WFA in astrocytes corroborates our previous findings that demonstrated that the potent inhibition of endothelial cell proliferation occurs through $G_{0}/G_{1}$ arrest at low nanomolar concentrations of WFA (18). This WFA mechanism of growth arrest would be in keeping with other reports that showed the pharmacological exploitation of the $G_{1}$ cell cycle inhibitor flavopiridol in a brain injury model of gliosis to target reactive astrocytes and glial cells in vivo (47). However, our attempts to gain more meaningful mechanistic insight of the GFAP targeting activity of WFA in Vim KO astrocytes have proved to be arduous. Vim KO cells have an intrinsic lower rate of cell proliferation compared with WT cells (48), as also evidenced by our data and reports showing Vim KO astrocytes being predominantly found in $G_{0}/G_{1}$ (49). Thus, because Vim KO astrocytes do not transition $G_{1}/S$ in response to serum stimulation they fail to respond to WFA despite potent down-regulation of soluble GFAP. This, and other aspects of Vim KO astrocytes that reveal their terminal fibroblastic differentiation after few rounds of passages, their heterogenous phenotypes, and eventual down-regulation also of GFAP expression (50) are other confounds we faced with this culture model. Nevertheless, the Vim KO mice consistently increased cyclin D3 expression in the retina upon injury. Thus, it was intriguing that WFA treatment further enhanced cyclin D3 expression and sustained PCNA levels in injured Vim KO retinas, corroborating the stimulation of the GFAP filament assembly mechanism with the maintenance of the cell proliferative response. This biological response is due to the in vivo selective targeting of WFA of certain higher molecular weight soluble GFAP species in Vim KO mice. Given that efficient targeting of all GFAP isoforms by WFA is exercised in the organ culture model of Vim KO retinas by high WFA concentrations ex vivo, it would be anticipated that a corresponding higher dose of WFA would down-regulate all GFAP isoforms in Vim KO retinas in vivo. This is evidently not achieved by 2 mg/kg of WFA. It would appear that down-regulation of multiple/all soluble GFAP isoforms induced by stress are required for effective inhibition of gliosis, which correlates with such an effect caused by the Rho-kinase inhibitor (51) and our findings made with WFA in WT mice.

The pharmacological total knock-out of soluble GFAP and vimentin to emulate the combined genetic deficiency of both IFs (Vim GFAP dKO) despite the encouraging results of increased neural regeneration and improvement of CNS functions in Vim GFAP dKO mice (15, 52) may come also with some

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**FIGURE 8.** WFA differentially regulates cyclin D3 and p27<sup>kip1</sup> expression in injured WT and Vim KO mouse retinas. WT and Vim KO mice were subjected to the alkali injury model and treated with 2 mg/kg/day of WFA or with vehicle by intraperitoneal daily injection for 7 days. Tissue sections of eyes from uninjured (Cont) and injured mice were stained separately with antibodies to either cyclin D3 (A) and p27<sup>kip1</sup> (B) and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) and assessed by epifluorescence microscopy. The bar in representative retinal images is 20 μm. GCL, ganglion cell layer; OXL, outer nuclear layer. Data are representative of two independent experiments (n = 12 mice). C, representative Western blot of uninjured (Cont), injured (Veh), and WFA-treated retinas isolated from WT and Vim KO mice probed with antibody to PCNA. GAPDH serves as control for loading accuracy (n = 2). The bar graph represents the optical density of PCNA protein bands normalized to GAPDH (y axis arbitrary units).
Withaferin A Targets GFAP

risk in trauma healing. For instance, Vim GFAP dKO mice have a high incidence of mortality when subjected to brain injury (9) and retinal Müller glial endfeet are also especially sensitive to physical injury (53). In contrast, we found that pharmacological delivery of 2 mg/kg of WFA causes knockdown of soluble vimentin and GFAP in injury healing WT mice to levels found in uninjured controls, and this WFA dose did not increase fragility of ocular tissues. Importantly, corneal angiogenesis was also simultaneously inhibited with 2 mg/kg of WFA in this chemical injury model, and without adverse effects on corneal resurfacing of denuded epithelium or on pre-existing limbal vasculature (10). Furthermore, we show that measurable levels of WFA (3 ng/μl) in serum are achieved 6 h after a single 10 mg/kg intraperitoneal injection (supplemental data); the 2 mg/kg dose of WFA thus provides a systemic anti-inflammatory dose sufficient to down-regulate transcription factor NF-κB activation (18, 54, 55). This posis is consistent with our previous finding that production of the potent inflammatory mediator TNF-α (56) is also blocked in vivo at this WFA dose (18). Our findings are also corroborated by a recent study that showed blockade of TNF-α release by a synthetic Rho kinase inhibitor promotes cell survival and reduction of retinal gliosis with down-regulated GFAP expression (51). Taken together, we illustrate here that the pharmacological knockdown of soluble type III IFs in activated retinal Müller glia is achievable in vivo. This strategy could be further exploited in animal models of retinal detachment, subretinal fibrosis, or proliferative vitreoretinopathy (57) to test the efficacy of WFA, or alternatively, to include WFA as an adjunct with standard anti-angiogenic therapy (58).

Last, it is known that multiple complex mechanisms, including phosphorylation (59), caspase cleavage (60), co-factor binding (61, 36), and alternate splicing (62) modulate IF stability and their assembly, where rapid subunit exchange through mechanisms still unknown also contribute to their dynamic behavior (63). Thus, characterizing the precise kinase-phosphatase balance that regulates the assembly-disassembly kinetics of GFAP in Müller glial cells and their regulation by WFA are clearly important goals for future studies. Importantly, such drug-induced augmentation of GFAP polymer assembly could also possibly be beneficial during the early stages of injury repair to protect neural tissues (64). This is because down-regulation of scar components immediately following injury, as opposed to later stages after injury, promotes increases in TNF-α levels and results in a poor healing outcome in the CNS trauma spinal cord injury model (65).

Collectively, our findings support the hypothesis that the critical function of vimentin as a facilitator of GFAP filament assembly (34) is relevant in this retinal model of gliosis and is validated using genetic and pharmacological tools to perturb vimentin expression. As further demonstrated in our study, WFA also independently binds soluble GFAP and down-regulates its expression, which opens up novel small molecule approaches to investigate orphan human genetic disorders linked to GFAP mutations, such as Alexander disease (66). Clearly, brain and spinal cord trauma victims face imminent reactive gliosis (67), for which, small molecule strategies exploiting the withanolide-based structure could be explored for therapeutic development.

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Withaferin A Targets Intermediate Filaments Glial Fibrillary Acidic Protein and Vimentin in a Model of Retinal Gliosis

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